METHOD FOR MEASURING THE APOPTOSIS

The invention relates to the field of biological test methods.

Apoptosis or programmed cell death (PCD) is a genetically controlled cellular suicide mechanism for selectively eliminating unwanted cells (1-4). PCD is an absolutely essential process in a number of biological processes, including embryonic and neural development, the regulation of the immune system, organogenesis, tissue homeostasis and the prevention of diseases such as tumour growth and virus infections. Apoptosis is characterised by blistering of the plasma membrane, shrinkage of the cells, condensation of the nucleus, endonucleolytic cleaving of genomic DNA into fragments of internucleosomal length and the formation of apoptotic bodies.

The methods currently available for investigating apoptosis are based on evaluating morphological changes at the cell level using light, electron or time-lapse microscopy in conjunction with fluorescent vital dyes, the use of annexin V, which can be used to monitor the loss of membrane phospholipid asymmetry during apoptosis (7), or they comprise assays for detecting DNA fragmentation by gel electrophoresis (8) or by *in situ* labelling of DNA strand breaks („nick-end labelling ") (TUNEL) (9).

However, most of these methods are either unsuitable for investigating the effect of genes which play a part in apoptosis, by transient transfection analysis, or,

in the case of the TUNEL method, too expensive and laborious.

The problem of the present invention was to provide a new method of measuring apoptosis which overcomes the disadvantages of the known methods.

This problem was solved by a process for measuring apoptosis which is characterised in that

A) a population of mammalian cells is transiently transfected

ai) with a plasmid containing a DNA sequence of interest which is to be investigated as to whether it or the polypeptide expressed thereby has a pro- or anti-apoptotic activity,

aii) or with a plasmid containing a DNA sequence of interest which is to be investigated as to whether, or by means of which substances, its pro- or anti-apoptotic activity or the activity of the polypeptide expressed thereby can be modulated,

b) and with a plasmid containing a DNA coding for a fluorescent marker protein,

B) in that the cells are incubated in a suitable nutrient medium, optionally in the presence of a test substance, until the DNA sequence of interest or the expressed polypeptide has exerted its potential effect on the apoptosis,

C) in that the cells are harvested and fixed so that the fluorescent protein remains in the cells,

while the DNA fragments formed during apoptosis are able to diffuse out of the cells,

D) in that the proportion of apoptotic cells is determined by measuring the DNA content,

E) in that the proportion of transfected cells is determined by measuring the cells having fluorescent marker protein,

F) and that by comparing the values obtained in steps D and E the proportion of apoptotic cells in the transfected subpopulation of the cells is determined.

The expression "DNA sequence of interest" (hereinafter also referred to as "apoptosis test gene") covers all DNA sequences which affect apoptosis directly or indirectly, as such or in the form of their translated products. Examples of genes which stimulate apoptosis are p53, bax, E1A, examples of apoptosis-inhibiting genes are bcl-2, bcl-x, E1B 19K, the latter group also including the so-called survival factors such as insulin-like growth factors (IGFs). Apoptosis genes of this kind and their activity have been described in summarising articles (e.g. 4, 23, 24).

The apoptosis test genes may be known or unknown genes or fragments thereof. By influencing apoptosis is meant both inducing and reinforcing as well as blocking and attenuating apoptosis.

The method according to the invention allows great variation, e.g. in terms of the markers used for determining the transfected cells and for the

apoptosis, in terms of the plasmids and method of transfection used for transfecting the cells.

The method according to the invention has as one of its essential elements a fluorescent marker protein which serves to indicate the transient transfection of the cells.

The preferred marker protein is Green Fluorescent Protein (GFP). GFP mutants, which are tailored for FACS analysis and are suitable for use within the scope of the present invention, are known from the literature. One example of a suitable GFP mutant was described in (10) ("enhanced Green Fluorescent Protein", eGFP); however, within the scope of the present invention, other mutants may also be used which satisfy the condition that they do not influence cell metabolism, they remain located at the intracellular level and they deliver a measurable fluorescence signal, and in particular they are measurable using current methods of fluorescent activated throughflow cytometry (Fluorescent Activated Cell Sorting (FACS)).

Apart from Green Fluorescent Protein (GFP) other fluorescent marker proteins may also be used. Examples include Blue Fluorescent Protein (BFP) (26) and Yellow Fluorescent Protein (YFP) (25). The properties mentioned above for GFP mutants are essential for the suitability of a marker protein for use in the method according to the invention.

Potential marker proteins and the type and quantity of the plasmids coding for them which are to be used in the assay as well as the most suitable transfection method can be tested as follows, for example: the plasmids coding for the marker proteins are transiently

transfected into mammalian cells, appropriately in the same cells and under the same conditions as are to be used for the method according to the invention. The suitability of the transfected marker proteins is determined by series of measurements in which the transfection efficiency and the efficiency of the reproducible measurement are determined by FACS analysis.

The marker used for the apoptosis is a DNA-binding stain, e.g. propidium iodide (PI), which causes a reduction in fluorescence in the apoptotic subpopulation (14-17). This method of detection is based on the principle that the genomic DNA in cells is broken down endonucleolytically during apoptosis. The small DNA fragments diffuse out of the cell; the reduction in the DNA content to less than twice the set of chromosomes ("sub-2N") is an indication of apoptotic cells.

The reduced fluorescence of PI in cells which are undergoing apoptosis results in the appearance of a characteristic fluorescence peak ("sub-2N-peak") in the area of the G_0/G_1 region of the cell cycle.

Instead of propidium iodide, other DNA-binding stains may be used. Examples of suitable stains of this type are commercially available, e.g. DAPI (4',6'-diamidino-2-phenylindole), acridine orange, ethidium bromide. The most suitable stain can be determined by stimulating cells to apoptosis and then determining by FACS or microscopic analysis whether apoptosis can be reproducibly measured with the candidate stain.

One of the advantages of the method according to the invention is that the fluorescence of the marker

protein and the DNA content can be measured simultaneously, preferably by FACS analysis. Suitable equipment is commercially available.

The invention is applicable to all mammalian cells which can be cultivated. It is a routine procedure for anyone skilled in the art to adjust the standard commercial FACS apparatus to different cell types.

For the transfection of the cells with marker gene on the one hand and the gene of interest on the other hand, all vectors which bring about efficient and reproducible expression in mammalian cells are suitable. Some of the numerous vectors available, including those which are commercially obtainable, contain regulatory sequences capable of achieving high expression rates in a number of mammalian cells. Examples include vectors which contain the CMV-(Cytomegalovirus), the SV40-(Simian virus), MSV (Moloney Sarcoma Virus)-promoter or other powerful promoters non-specific to cell type.

Identical or different vectors may be used as carriers for the marker gene and gene of interest; depending on the type of cell it may be advantageous to use vectors with different promoters, in order to avoid competition between the promoters during the transcription.

With regard to the transfection methods the invention is not subject to any restrictions; theoretically, all the methods known for the transient transfection of mammalian cells can be used, e.g. calcium phosphate, commercially obtainable cationic lipids such as lipofectamine or transfectam, methods based on receptor-mediated, Adenovirus-aided endocytosis, as described e.g. in WO 93/07283, for example using

polyethyleneimine and psoralene/UV inactivated Adenovirus, as described in (21). The transfection method can be optimised, using series of tests in which the transfection conditions, type of cell, nutrient medium etc. are varied, by transfecting with a fluorescent marker protein and determining the expression of the protein by FACS analysis. The optimised conditions for the marker protein are used for the co-transfection with the gene of interest.

After the transfection the cells are incubated in a suitable nutrient medium which is adapted to the particular type of cell. The cells may optionally be stimulated to apoptosis, particularly if the apoptosis test gene is to be investigated for any inhibition of apoptosis. Suitable apoptosis stimuli are known from the literature and commercially available; examples include staurosporin, daunomycin and etoposide. The incubation conditions and the suitability of an apoptosis stimulant are determined in preliminary trials. It is essential for the incubation, particularly its duration, that apoptosis has taken place to an extent which enables any change to be measured by measuring equipment, e.g. by FACS analysis.

The fixing step which is carried out after the incubation is essential to carrying out the process according to the invention.

The essential requirement for the fixing is that the conditions are such that the small subgenomic DNA fragments (internucleosomal fragments, i.e. those with a size of about 200 bp or a multiple thereof) occurring on apoptosis are able to diffuse out of the apoptotic cells, but at the same time the fluorescent marker protein remains in the cell. With the methods available

up till now it was not possible to combine these measurements as the demands made on the fixing with respect to measuring the fluorescent marker protein on the one hand and measuring the DNA content of the cells on the other hand were diametrically opposed and therefore seemed to be irreconcilable. The present invention makes it possible for the first time to carry out both measurements in the same cell population using a suitable fixing step.

In order to determine suitable fixing conditions the following procedure is preferably followed: first of all, the optimum fixing conditions for measuring the fluorescence of the marker protein on the one hand (strong fixing) and the optimum fixing conditions for measuring the DNA content of the cells on the other hand (weakest possible fixing) are determined independently of each other. Starting from the conditions with which the maximum measurements are obtained, the fixing conditions are modified in terms of the reagents (fixing reagent, salts, buffer), the concentration thereof and the fixing time in such a way that the efficiency is affected as little as possible when the two measuring operations are carried out simultaneously.

Preferably, the primary fixing is carried out with paraformaldehyde and the subsequent treatment (secondary fixing/permeabilisation) with ethanol; this treatment has proved most suitable in the tests performed. The primary fixing using 1 to 4 % (w/v), particularly 2 % paraformaldehyde takes place in an isotonic buffered saline solution. Standard solutions are suitable, such as 100 mM NaCl, 3 mM $MgCl_2$, 300 mM saccharose as well as standard commercial physiologically acceptable buffers.

Instead of paraformaldehyde it is also possible to use other reagents such as those which are conventionally used, e.g. in immunohistochemistry. Examples of common fixing agents which can be found in the relevant textbooks (27) include formaldehyde or chloroform/acetone.

Instead of ethanol, which has proved particularly suitable under the conditions selected in the tests carried out for the secondary fixing following on from the primary fixing with paraformaldehyde, it is theoretically possible to use other reagents which render the cell membrane slightly permeable, such as detergents, for example.

The transient expression of genes which modulate apoptosis, for example members of the Bcl family or components of the survival factor signal transduction, and the subsequent quantitative analysis of apoptosis using the method according to the invention make it possible to test chemical compounds to see whether they are capable of specifically influencing the function of apoptosis-modulating genes.

The method according to the invention can be automated by a suitable adaptation of apparatus, e.g. the preparation of samples and the FACS analysis, which makes it suitable for carrying out measurements on a large scale, e.g. in High Throughput Screening methods.

The method in this form is used in the identification of pharmaceutically active substances which are able to modulate apoptosis as a function of the expression of certain genes (apoptosis test genes). The gene whose effect on apoptosis is supposed to be modulated by the

test substance is transiently transfected into test cells and the test cells are incubated with a test substance from a range of substances available. The modulating effect of a test substance on the activity of the test gene is measured directly using measuring instruments.

Methods of this kind can be used for the following screening applications:
a) searching for inhibitors of survival factors and their signal transduction, as well as inhibitors of anti-apoptotic gene products in tumour cells; b) searching for chemicals which, synergistically with chemotherapy, inhibit certain survival factors and their signal transduction in tumour cells; c) searching for chemotherapeutic agents which act synergistically with the inhibition of survival factors.

In one embodiment, the method according to the invention is used in a screening process to investigate the effect of survival factors inherent in tumour cells (receptor ligands such as IGF-I, IGF-II, FGFs (Fibroblast Growth Factors), PDGFs (Platelet Derived Growth Factors) on apoptosis, as mediated by the activation of the corresponding receptors by these factors and the subsequent signal cascade. The assay method can be used in a screening in order to modulate the activity of natural, known or possibly yet to be identified survival factors with respect to a tumour therapy in the course of which the apoptosis of tumour cells is to be intensified. The aim of a process of this kind is particularly to identify substances which, with regard to apoptosis, work synergistically with the inhibition of tumour-specific survival factors.

To detect synergistic effects of this kind the following procedure may be used:

Test cells are prepared, starting from tumour cells, in which the survival factor function is inhibited by introducing into the cells, and expressing, DNAs coding for dominant-negative versions of receptors of the survival factors or for dominant-negative signal transmission molecules of such receptors. Examples of receptors are the IGF-1 receptor (29), FGF receptors (30), PDGF receptors (31), receptors of the EGF-growth factors (32; EGF receptor, Her-2/~~Hew~~Neu/ErbB-2, ErbB-3, ErbB-4). Examples of signal transmission molecules are Ras, Raf, phosphoinositol(3)kinase (=PI(3)-kinase), MAP kinases, type B and type C protein kinase, phospholipase C, and also adapter molecules such as Shc, Grb-2 (33; 34; 35; 36; 37).

Suitable receptor mutants are characterised in that the functional domains of the receptor are modified so that the receptor does indeed bind the ligand but this binding no longer results in the activation of the signal cascade. In the case of IGF-1R the modification comprises the complete absence of the receptor kinase domain or a mutation of the ATP binding site (28). Signal transmission molecules can be modified by inactivating the domains needed for transmission of the signal, e.g. the catalytic domain of an enzyme or the protein binding site of an adapter molecule, by mutation.

The mutants provided for use in a screening process are optimised in preliminary tests for their apoptosis-inducing or -increasing activity in test cells (e.g. fibroblast cell lines which have been made factor-dependent by transfection with the appropriate wild-type receptor) by current methods, in series of tests, by transfecting the mutants into the test cells and measuring the extent of the apoptosis of the test cells using the method according to the invention. The particular functional domains of the mutants are further modified, if necessary, by current molecular biological methods, until an optimum inhibition of the wild-type receptor and its subsequent signal transmission and hence a maximum level of apoptosis of the test cells has been achieved.

In the screening, the test cells are incubated with known chemotherapeutic agents or with substances from a pool which are to be investigated for their potential chemotherapeutic activity and the effect on apoptosis is investigated by the method according to the invention. In particular a screening operation of this kind sets out to find any synergistic activity between the inhibition or absence of the survival factor function in tumour cells and known chemotherapeutic agents or potentially chemotherapeutically active substances.

In order to use the screening process to find substances which exhibit synergism with the inhibition of the survival factor function specifically for certain types of tumour, cells derived from different

types of tumour may be used in a parallel screening test, under otherwise identical experimental conditions.

The cells used as control cells for the specificity of the synergistic activity between the absence of the survival factor function and chemotherapy are cells which are naturally lacking the particular survival factor function the inhibition of which is to be investigated in the assay.

However, theoretically, it is also possible to look for substances which increase the activity of apoptosis-inducing or -increasing molecules. Examples include members of the TNF receptor family (TNF receptors, Fas) and molecules of their signal transmission pathways (caspases). The test principle is exactly the same as for the apoptosis-inhibiting survival factors, except that wild-type or constitutively active versions of these apoptosis molecules are expressed in the tumour cells.

Thus, the main focus of the screening is the search for synergisms which will lead to an increase in tumour cell apoptosis. This provides the prerequisite for therapeutic approaches in which, at the same time as the tumour cell survival function is inhibited, the dosage of chemotherapeutic agents can be significantly reduced without affecting the success of the treatment. The reduction in the chemotherapeutic dose has crucial advantages for the patient, as the toxic side-effects of the chemotherapy can thus be greatly reduced.

Within the scope of the present invention, the method according to the invention was used to investigate whether the signal mediated by the survival factor IGF-II which brings about the survival of β-tumour cells is transmitted by the IGF receptor. For this purpose, a DNA coding for dominant-negative version of the human IGF-1 receptor (dnIGF-1R), which has an amino acid substitution in the ATP-binding site (28), is transiently co-transfected into wild-type β-tumour cells with a plasmid carrying Green Fluorescence Protein (eGFP). The cells were then incubated with apoptotic stimuli and/or growth factors, harvested, fixed and stained with propidium iodide in order to determine the DNA content. Using Facs analysis, single transfected cells expressing eGFP were detected and in this population the apoptotic cells were identified by their DNA content of less than 2 N. It was found that the transfection with plasmids which code for the dnIGF-1R leads to a dramatic rise in apoptosis both in untreated wild-type β-tumour cells and in such cells treated with daunomycin or etoposide. It was found that dnIGF-1R intensifies the apoptosis in β-tumour cells with almost the same efficiency as the Adenovirus-E1A protein, one of the most powerful apoptosis-inducing gene products there is. Thus, using the assay method according to the invention, it was possible to show that tumour cells react more sensitively to apoptotic stimuli if the IGF-1R signal transmission is interrupted and that, like IGF-II-deficient tumour cells, they exhibit greater sensitivity to chemotherapeutic agents.

Moreover, the method according to the invention can be used to investigate known genes as to whether and to what extent they modulate apoptosis in different types of cells.

Another use for the method according to the invention is the expression cloning of genes which modulate apoptosis. For this, a complete cDNA expression library is transiently transfected into cells. The method according to the invention is capable of measuring the influence of gene expression within 24 to 48 hours. It is therefore possible to analyse and isolate cells while they are still alive. For this purpose the method is modified by using FACS sorting to isolate single cells which deviate from an apoptosis background which is to be determined. The plasmids transfected into these cells are isolated, amplified and selected in further transfection processes. Plasmids which contain an apoptosis-modulating gene are thus isolated. The corresponding genes are then characterised by sequencing and other studies of expression and function.

To validate the method according to the invention, first of all, in Example 1, established tumour cell lines were used which had been transfected on the one hand with a GFP plasmid and on the other hand with a plasmid containing a pro-apoptotic or an anti-apoptotic gene sequence, or a control plasmid. After the transfection the cells were treated, after a period of rest, with an apoptotic stimulus (control cells remained untreated). Then the detached cells were collected and combined with the trypsinised adherent cells, washed and fixed. After being washed the cells were divided up to make it possible to compare the method according to the invention with the conventional

TUNEL method which uses fluorescent Cy5-dCTP (5-amino-propargyl-2'-deoxycytidine 5'-triphosphate coupled to Cy5 fluorescent stain).

It has been found that the method according to the invention reliably detects the expected pro- or anti-apoptotic effect of the gene products and that the addition of the apoptotic stimulus intensifies the effect observed. Although the sensitivity of the method according to the invention under the conditions selected was slightly less than that of the TUNEL method, the standardised apoptosis value, expressed as the ratio of the maximum apoptosis values of the particular assay achieved with the pro-apoptotic gene, was virtually identical. Compared with the TUNEL method the method according to the invention has the advantage of speed, simplicity and cheapness.

The versatility and reliability of the method according to the invention were confirmed by the use of an untransformed rat fibroblast cell line which reacts less to apoptotic stimuli than the established tumour cell lines.

The method according to the invention makes it possible to establish the potential rôle of a gene product in apoptosis rapidly, effectively and reproducibly.

According to another aspect, the invention relates to a kit for carrying out the process simply as a routine procedure.

A kit of this kind will expediently contain the following components in a number of separate containers:

a) one or more components required for the transfection;
b) a plasmid containing the sequence coding for the fluorescent marker protein;
c) an empty vector for inserting the DNA sequence of particular interest and for control measurements;
d) the primary fixing solution, e.g. paraformaldehyde solution;
e) the secondary fixing/permeabilising solution, e.g. 70 % ethanol;
f) washing solution(s);
g) a DNA-binding stain.

Preferably, the kit contains polyethyleneimine and psoralen/UV-inactivated Adenovirus as transfection components.

Example 1

For this Example, established tumour cell lines (βTC and βHC) were used, derived from β-cell tumours (15) in transgenic mice, in which the regulatory region of the insulin gene (Rip) specifically induces the expression of the large T-antigen of Simian virus 40 (Tag) in the β-cells of pancreatic islets (16).

About 80,000 cells were seeded into a 6 cm well in a 6-well culture dish and cultivated in DMEM, supplemented with 10 % FCS (v/v), 2 mM glutamine, 100 International Units of penicillin and 100 µg/ml of streptomycin, until 70 % confluence was achieved. The cells were transfected with 1 µg of a plasmid coding for eGFP ("enhanced GFP"; pEGFP-C1; Clontech) together with 1 µg of a control plasmid (pMEX; (22)), a pCMV plasmid containing the pro-apoptotic Adenovirus gene E1A or a

pCMV plasmid containing the anti-apoptotic Adenovirus gene E1B-19K (17, 18) using 10 µl of LipofectAMINE (GIBCO-BRL) in accordance with the manufacturer's recommendations. After the transfection the cells were left to stand for 16 h in complete medium, then the cells were either left untreated or treated for a further 16 h with an apoptotic stimulus (800 ng/ml of staurosporin; Sigma) (19, 20). 32 h after the transfection the detached cells were combined with trypsinised, adherent cells, washed twice with 4 ml of PBS and fixed at ambient temperature for 30 min (2 % paraformaldehyde, 100 mM NaCl, 300 mM saccharose, 3 mM $MgCl_2$, 1 mM EGTA (ethyleneglycol-bis-(2-aminoethyl)-tetraacetic acid), 10 mM PIPES (piperazine-N_1N^1-bis[z-ethanesulphonic acid]) pH 6.8). Then they were washed twice with 4 ml of PBS and fixed for 14 h in ice-cold 70 % EtOH.

After the fixing, the cells were washed twice with 4 ml of PBS and divided up. One half of the sample was treated with RNase A (Sigma, St. Louis, USA)(50 µg/ml) in PBS for 30 min, washed twice with 4 ml of PBS and, 30 min before the FACS analysis, stained with propidium iodide in PBS (PI; 50 µg/ml; Sigma, St. Louis, USA). The other half of the sample was incubated for 1 hour at 37°C with 50 µl of TdT reaction mixture (terminal deoxynucleotidyl transferase; Boehringer Mannheim; 200 mM potassium cacodylate, 25 mM of Tris-HCl, pH 6.6, 0.25 mg/ml bovine serum albumen, 1 mM $CoCl_2$; 0.25 nmol FluoroLink Cy5AP3-dCTP [Amersham], 12.5 units of TdT), washed twice with 4 ml of PBS, treated with RNase in PBS (50 µg/ml) for 30 min, washed twice with 4 ml HBS (from this step onwards HBS was used instead of PBS because DAPI has a tendency to produce microprecipitates in PBS), stained with DAPI in HBS (10 µg/ml; Sigma) for 20 min and analysed using a

Becton Dickinson FACS Vantage apparatus. The FACS analysis of the PI-stained cells was carried out with a Becton Dickinson FACScan apparatus fitted with a so-called "doublet discrimination module", with which cell aggregates are discriminated by calculating the pulse width ~~and pulse width~~. The results of the tests are shown in Fig. 1. Fig. 1A shows the number of apoptotic βHC 13T tumour cells (% apoptosis) in the entire eGFP-positive cell population. The black bars indicate the determination of the sub-2N DNA content (GFP/PI); the white bars indicate the incorporation of fluorescent Cy5AP3-dCTP during the TdT reaction (GFP/TUNEL). The addition of staurosporin is shown. An excitation wavelength of 488 nm was used for eGFP and PI, an excitation wavelength of 647 nm was used for Cy5 and UV of a wavelength range of 51 - 364 nm was used for DAPI. The emission fluorescence was collected using a 530/20 nm narrow band filter for eGFP, a 610 nm blocking filter for PI, a 675/20 nm narrow band filter for Cy5 and a 424/44 narrow band filter for DAPI. Doublets were excluded by means of pulsed processing. eGFP-expressing cells were selected and analysed for Cy5- or PI-fluorescence. The data were analysed using CELLQuest software (Becton Dickinson). Each bar represents the average of 3 transfections, standard deviations are indicated by error bars. Each measurement comprised 40,000 total events, selected according to size and single cells. The transfection efficiency was 20-30 %.

Fig. 1B shows the standardised percentage of apoptosis for the various constructs. The apoptosis index was standardised using the following function: (% apoptosis in X/% apoptosis in eGFP-C1/E1A) x 100. The apoptotic index was standardised for each of the detection

methods used and for each subsequent transfection treatment (+/- staurosporin).

Example 2

In this Example an untransformed rat fibroblast cell line designated Rat1A was used. The cells were transiently transfected using either LipofectAMINE, as described in Example 1, or polyethyleneimine (PEI 2000)-DNA-Adenovirus complexes (WO 93/07283). Moreover, regarding the treatment of the cells and determination of apoptosis, using the process according to the invention on the one hand and the TUNEL method on the other hand, the procedure was exactly as described in Example 1. A comparison of the different transfection methods and methods of measuring apoptosis is shown in the Table. Each value represents the average of 3 transfections; the standard deviation is given (s.d.).
The efficiency of the transfection methods was 25-30 %.

Example 3

In this Example wild-type β-tumour cells (15) were used. The dominant-negative IGF-1 receptor construct used, which is under the control of the CMV promoter and in which the codon 1003 in the ATP binding site is mutated from lysine to alanine, was described by (28). As described in the previous Examples, wild-type β-tumour cells were seeded at a density of 80,000 cells in triplicate in 6-well culture dishes. 24 h later the cells were co-transfected with pEGFP-C1 and a control plasmid (Fig. 2: pMEX/ctr) or with pEGFP-C1 and an expression plasmid, coding either for Adenovirus-E1A

(Fig. 2: E1A), Adenovirus-E1B-19K (Fig. 2: E1B-19K) or the dominant-negative IGF-1R. 36 h after transfection, daunomycin (Fig. 2: shaded bar) or etoposide (Fig. 2: white bar) was added to the culture medium in a concentration of 1 μM or 10 μM. Transfected but untreated cells are indicated in Fig. 2 by black bars. 12 h after treatment with daunomycin or etoposide the cells were harvested, fixed and treated with propidium iodide, as described in the previous Examples. The apoptotic cells were also determined using the methods described above. For each measurement 40,000 events were collected; the transfection efficiency was 25-30 %. The standard deviation is indicated by error bars.

Table

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Trans-fected Construct	LipofectAMINE			
	Propidium iodide		TUNEL	
	% apoptosis (s.d.)	% apoptosis standardised	% apoptosis (s.d.)	% apoptosis standardised
pMEX (Ctr)	3.3 (0.7)	68.8	6.5 (0.6)	71.4
pE1B-19K	1.4 (0.3)	29.2	2.2 (0.5)	24.2
pE1A	4.8 (0.4)	100	9.1 (2.6)	100
	PEI / Adeno			
pMEX (Ctr)	1.2 (0.2)	52.2	7.5 (0.3)	52.1
pE1B-19K	0.7 (0.1)	30.4	5.2 (0.8)	36.1
pE1A	2.3 (0.6)	100	14.4 (2.7)	100

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